Simultaneous multichannel nonlinear imaging: combined two-photon excited fluorescence and second-harmonic generation microscopy

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ABSTRACT

Simultaneous two-photon excited fluorescence (TPF) and second-harmonic generation (SHG) imaging is demonstrated using a single femtosecond laser and a scanning microscope. This composite nonlinear microscopic technique was applied to imaging DNA and chromosomes, and it was shown that the two different interaction mechanisms provide complementary information on the structure and nonlinear properties of these biological materials beyond that achievable using either TPF or SHG imaging alone. The use of separate modes of detection, in reflection and transmission respectively, and the simultaneous nature of the acquisition of the two images allows pure TPF and SHG images in precise registration to be obtained.

Key words: nonlinear imaging, two-photon microscopy, fluorescence, second-harmonic generation

1. INTRODUCTION

Confocal fluorescence laser scanning microscopy has been widely used for three-dimensional high-resolution optical imaging of biological specimens. More recently, several nonlinear optical microscopies have been developed including those based on two-photon excitedfluorescence,[1,2] three-photon excited fluorescence,[3] second-harmonic generation[4,5] and third-harmonic generation.[6,7] These nonlinear microscopies have been shown, to various extents, to be valuable techniques for biomedical imaging[2,8,9] and their use is likely to become more wide-spread particularly with the increasing availability of turn-key solid-state femtosecond laser sources. These microscopies are based on the interaction between the high peak power density that exists at the laser focus and the nonlinear optical properties of the specimen in the focus region. The spatial variation of this interaction in the focus region gives a "soft-aperture" effect which leads to an inherent optical sectioning capability without the need for a confocal pinhole. For example, in two-photon imaging, the intensity of the signal produced is proportional to the square of the fundamental incident intensity leading to a reduction in the size of the effective point-spread-function by a factor of $\sqrt{2}$.[10,11] The interest in these techniques stems partly from the greater penetration and hence imaging depth possible with the longer (usually near-infrared) wavelengths used, the reasonably high interaction efficiency especially for two-photon excited fluorescence, and the improved optical detection efficiency arising from the fact that a confocal pinhole is not required. While two-photon excited fluorescence (TPF) and second-harmonic generation (SHG) microscopies have been demonstrated using CW[4,12] and long-pulse[13] lasers, femtosecond sources are generally more desirable because the effect due to a given pulse varies inversely with the square of the laser pulse-width for pulsed excitation.

Although TPF and SHG imaging systems are optically similar, the nature of the interactions and the contrast-generating mechanisms are very different. TPF is a resonant process involving simultaneous absorption of two low energy photons to cause molecular electronic excitation followed by fluorescent emission with an angular distribution determined by the molecular configuration (symmetry and orientation) and a fluorescence decay time determined by the electronic upper-state lifetime. On the other hand, SHG is a non-resonant virtually instantaneous process giving rise to harmonic radiation in the forward direction. Therefore, TPF is incoherent whereas SHG is coherent. Obviously, TPF can be detected over the emission bandwidth of the fluorophore whereas SHG radiation occurs only at the second harmonic of the excitation laser frequency.

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So far, all of the 3D optical imaging techniques, whether they involve single-photon or multiphoton excitation, have been applied as single microscopic techniques. Even when more than one two-photon technique is used in a study,[14] this has involved two separate single-channel measurements made at different times with different experimental parameters. However, there is also the possibility of imaging a specimen using two or more imaging modes (or channels) simultaneously in time. Such a multichannel imaging approach may be particularly attractive for nonlinear microscopy where the laser pulse may interact with several nonlinear optical properties of the specimen and the resulting optical signals may be detected separately to form simultaneous composite nonlinear optical images of the specimen. Furthermore, a simultaneous imaging capability would be useful for time-series studies and in cases where photodamage must be minimised or where specimen changes or drift occur during the scan period. In this paper, we demonstrate high-resolution TPF and SHG imaging performed simultaneously in time using a two-channel synchronized detection method. The approach is illustrated by imaging DNA and chromosomes using a modified commercial confocal microscope. We also show that the combination of TPF and SHG imaging provides information about the specimen that is beyond that provided by either TPF or SHG imaging alone.

2.MATERIALS AND METHODS

Two-photon microscopy was carried out using a modified inverted Leica TCS NT confocal microscope coupled to a Coherent Mira 900 femtosecond Ti:sapphire laser (~150 fs pulse-width, 720-860 nm) pumped by a Coherent 5W Verdi solid-state laser. For this laser pulse-width, an adjustable group velocity dispersion (GVD) precompensation arrangement was not necessary.[15] This microscope is of the beam-scanning type with an acquisition rate of up to 4 images (512 x 512 pixels) per second. All images were taken with a x40/0.75NA air objective. An important capability of this system is that it can operate simultaneously in both transmission and reflection modes. Fig. 1 shows a schematic of the system for simultaneous TPF/SHG

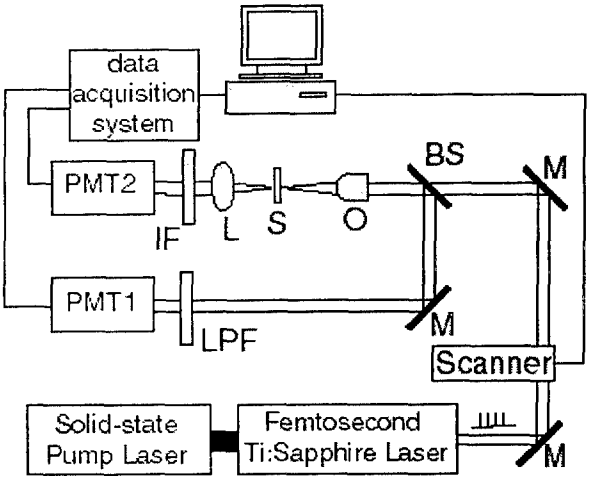

Fig. 1. Experimental arrangement for simultaneous TPF/SHG imaging. BS, beamsplitter; IF, 400 nm interference filter; L, lens; LPF, long-pass filter; M, mirror; O, objective; S, specimen.

imaging in which fluorescence was detected in reflection and second-harmonic light was detected in transmission. For the fluorescence channel, a broadband dichroic was used and the fluorescence detection bandwidth determined by a long-pass filter with a cutoff wavelength ~450 nm. For the SHG channel, the 800 nm fundamental beam was excluded from the detector using a 400 nm interference filter with a bandwidth of 20 nm FWHM and a rejection ratio at 800 nm of $>10^5$. In order to maximise the sensitivity, particularly for the unstained specimens, no confocal pinhole was used.

The observed resolution for both TPF and SHG modes was ~0.5 μm, which is close to the theoretical value of $0.61\ \lambda / NA\sqrt{2}$ = 0.46 μm. We emphasise that in the simultaneous TPF/SHG mode, the TPF and SHG images are acquired essentially simultaneously with synchronization to within the aperture time (~2 μs) of the sample-and-hold circuitry. The time registration is therefore better than the pixel dwell time.

3.RESULTS AND DISCUSSION

In order to demonstrate multichannel TPF/SHG imaging, we chose biological specimens that are well-defined and well-known, for example DAPI-stained cultured onion root cells in various stages of mitosis, TPF and SHG images of which are shown elsewhere.[16] These cells do not give strong natural fluorescence and so are best imaged using a fluorescent labelling dye such as DAPI. Although this enables straighforward TPF imaging, SHG imaging of specimens labelled with DAPI is complicated by the fact that the second-harmonic wavelength (~400 nm) is within the fluorescence emission band of DAPI (~390 - 600 nm) and so may be overwhelmed by DAPI fluorescence.

Biological materials are attractive specimens for SHG imaging because biomolecules typically have large molecular anisotropy and second-order nonlinearity. Apart from the obvious interest in DNA, this biomolecular system is of particular interest here since SHG may be enhanced by pseudo-phasematching resulting from the extended double-helix structure. This can then give a relatively strong contrast mechanism in SHG imaging and the prospect of obtaining microscopic nonlinear information about DNA without the complications associated with additives such as fluorescent labels. Another sample which has been used is herring sperm DNA.[16]

In order to achieve simultaneous TFP/SHG imaging, it is desirable to use an unstained specimen which can yield a suitable level of natural fluorescence. We produced reflection TPF and transmission SHG images obtained simultaneously on precisely the same area and at the same depth for a specimen comprising *B. Tyroni* fruit fly polytene chromosomes. In these there are some features, which are in almost perfect registration, that appear common to each image and others that are clearly unique to one image or the other. Nevertheless, the fluorescence and harmonic images are clearly both qualitatively and quantatively distinct, and the two images represent essentially pure fluorescence and harmonic images. Therefore, the simultaneous acquisition of TPF and SHG images clearly provides a means of obtaining new and complementary information about this specimen.

Although we used a non-confocal detection method for both TPF and SHG imaging in order to maximise sensitivity, improved resolution can be obtained by using a confocal pinhole albeit at the expense of signal level.[17, 18] The approach used here could also be extended by using multiple detectors and the unsharp masking technique.[19] Since the 3D OTF for TPF shows negative values for large pinhole sizes,[20] the resolution can be further improved by subtracting the image formed with an open pinhole from that formed with a small pinhole. Therefore, an example of a simple layout for multichannel two-photon microscopy with enhanced spatial resolution might consist of two independent fluorescence detection channels (with and without pinholes) in either a reflection configuration, as used here, or in a transmission mode using dichroics to separate the fluorescence signals from harmonic signals which are best detected in transmission in this case. In this arrangement, differential processing of the separate images from the multiple detectors should yield a useful resolution enhancement. We also note that Klar and Hell[21] have recently demonstrated resolution beyond the diffraction limit by using a UV pulse to create a confined region of excited molecules in a specimen and a spatially different infrared pulse to quench fluorescence from the outer part of the focus region through stimulated emission.

3.CONCLUSIONS

We have demonstrated TPF and SHG imaging both separately and simultaneously. Furthermore, this has been achieved using a commercial femtosecond laser and a commercial confocal microscope together with only a few additional components. We believe that the simultaneous use of two or more multiphoton microscopic techniques will develop as an important new direction in 3D optical microscopy particularly in biomedical and materials applications where the use of several multiphoton interactions can provide greater insight into the physico-chemical properties of the sample. We envisage that in the future, microscopists may use a single flexible pulsed-laser scanning microscope which can be suitably reconfigured to study specimens using simultaneous detection of two or more multiphoton interactions with either confocal or non-confocal detection. Such a multichannel or multifunction optical microscope would be particularly useful for applications involving specimens with complex structure and subtle physico-chemical properties.

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